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SUSCEPTIBILITY

Implications of the EUCAST Trailing Phenomenon in *Candida tropicalis* for the *In Vivo* Susceptibility in Invertebrate and Murine Models

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ABSTRACT *Candida tropicalis* isolates often display reduced but persistent growth (trailing) over a broad fluconazole concentration range during EUCAST susceptibility testing. Whereas weak trailing (<25% of the positive growth control) is common and found not to impair fluconazole efficacy, we investigated if more pronounced trailing impacted treatment efficacy. Fluconazole efficacy against two weakly (≤25% growth), two moderately (26% to 50% growth), and one heavily (>70% growth) trailing resistant isolate and one resistant (100% growth) isolate were investigated *in vitro* and *in vivo* (in a *Galleria mellonella* survival model and two nonlethal murine models). *CDR1* expression levels and *ERG11* sequences were characterized. The survival in fluconazole-treated *G. mellonella* was inversely correlated with the degree of trailing (71% to 9% survival in treatment groups). In mice, resistant and heavily trailing isolates responded poorly to fluconazole treatment. *CDR1* expression was significantly higher in trailing and resistant isolates than in wild-type isolates (1.4-fold to 10-fold higher). All isolates exhibited *ERG11* wild-type alleles. Heavily trailing isolates were less responsive to fluconazole in all *in vivo* models, indicating an impact on fluconazole efficacy. *CDR1* upregulation may have contributed to the observed differences. Moderately trailing isolates responded less well to fluconazole in larvae only. This confirms clinical data suggesting fluconazole is effective against infections with such isolates in less severely ill patients and supports the current 50% growth endpoint for susceptibility testing. However, it is still unclear if the gradual loss of efficacy observed for moderately trailing isolates in the larva model may be a reason for concern in selected vulnerable patient populations.

KEYWORDS EUCAST, *Galleria mellonella*, trailing, azoles, fluconazole, *in vivo*

Candida tropicalis is among the five most common causes of invasive candidiasis and is associated with certain geographic regions of the world (e.g., Asia-Pacific and South and Latin America) and certain underlying conditions, including leukemia and chronic lung diseases (1–5). *C. tropicalis* candidemia carries a high mortality rate (6). Trailing growth has been described since the early days of broth dilution antifungal susceptibility testing. It was originally defined according to the original CLSI broth microdilution method (NCCLS M27-A), when the MIC was determined at 48 h (7). An evaluation of clinical and *in vivo* azole response rates showed that isolates resistant at 48 h, but susceptible at the 24-h reading, responded to treatment (7–9).

Current EUCAST and CLSI reference methods recommend that MIC values are read after 24 h using a 50% inhibition endpoint compared to that of a drug-free control (10, 11). EUCAST has no established definition for trailing, but laboratories in Europe have

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reported that a proportion of several *Candida* species, and especially *C. tropicalis*, display growth inhibition of only 50% to 80% over a broad concentration range, defined as trailing growth, when using the 24-h EUCAST broth microdilution method (12–14). However, the magnitude of the problem is largely unknown. Different definitions have been used with respect to proportional growth compared to the growth of a control (e.g., heavy trailers show >15% residual growth in one study but 25% to 49% in another) (12, 13). Methodological issues, including pH value and incubation time, were shown to impact the degree of resistance (15–17), and some authors report more trailing with the EUCAST method than with CLSI at 24 h (22% versus 1.7%) (18). In *Candida albicans*, variable regulation of drug resistance genes was observed for isolates trailing by the 48-h CLSI method (19), and various drug resistance mechanisms have been implicated in azole resistance in *C. tropicalis* (20–22). Yet, it is unknown if trailing is related to the variable regulation of epigenetic traits or resistance mechanisms.

In our experience and among 81 Danish *C. tropicalis* blood isolates in the nationwide surveillance program from 2012 to 2015, most trailing *C. tropicalis* isolates displayed 10% to 25% residual growth. Severe trailing isolates with residual growth of approximately 50% and classified as nonsusceptible constituted a minority (7.4%) (14). Difficulties in MIC determination persist particularly for isolates with trailing growth close to the 50% endpoint. Furthermore, it remains unclear if all trailing isolates are truly as susceptible *in vivo* as their nontrailing counterparts. The limited data from the CANDIPOP study demonstrated fluconazole treatment efficacy in a subset of patients with *C. tropicalis* displaying weak to strong trailing (13). However, the very low 30-day mortality rate (~10%) among the 21 initially fluconazole-treated patients in this analysis compared to the overall accumulative 30-day mortality (29% [15/51]) of all included patients with *C. tropicalis* suggested that a selected (less severely ill) subset of patients may have been receiving fluconazole (13, 23).

We were concerned if the conclusions drawn from earlier animal studies for isolates trailing only after 48 h might not apply to the minority of isolates with residual growth close to the 50% inhibition endpoint. We therefore further investigated the *in vitro* and *in vivo* efficacy of fluconazole against *C. tropicalis* isolates trailing after 24 h in EUCAST antifungal susceptibility testing. Furthermore, the sequences of *ERG11* in the included isolates and the *CDR1* expression levels were determined. The *Galleria mellonella* model was employed, as it has proven to be a suitable model to study *C. tropicalis* virulence and response to treatment, including to fluconazole (24–26). The *G. mellonella* model has, however, not been fully validated for *in vivo* studies; thus, the isolates were further examined in a murine model of immunocompetent and immunocompromised animals.

(An abridged data set from the *Galleria mellonella* experiments was presented as a poster at the 7th TIMM conference in Lisbon in 2015.)

RESULTS

***In vitro* characteristics of the selected isolates.** The weakly trailing isolates CT-WT1 and CT-WT2 were consistently categorized as susceptible by EUCAST and displayed mean trailing below 25% (Table 1 and Fig. 1). In contrast, the trailing isolates CT-TR1 and CT-TR2 were not clearly designated susceptible or resistant due to variations in endpoint reading. Two isolates were consistently categorized as resistant. One of these, the CT-TR-R isolate, displayed a reduced but persistent growth (>70% compared to the growth control) over the entire fluconazole concentration range, whereas CT-R displayed no trailing growth in the ≥4-mg/liter concentration range (Fig. 1). The degree of trailing was pH dependent and increased when lowering the pH, concomitantly with a 2- to 3-dilution-step increase of MIC values (Table 2). An expression analysis of the ABC-transporter *CDR1* revealed an approximately 10-fold upregulation in CT-R compared to that in both susceptible isolates CT-WT1 and CT-WT2, consistent with its categorization as a resistant isolate. Since the *CDR1* expression data in CT-WT1 and CT-WT2 were similar, they were merged in Fig. 2. A significant upregulation of *CDR1* in isolates with moderate to high trailing (CT-TR1, CT-TR2, and CT-TR-R)

TABLE 1 Isolate characteristics, including fluconazole MIC values and percentage of included isolates designated trailing

Isolate	MIC (mg/liter)[a]			Trailing (%)	
	Range ($n = 7$)	Mode	Geometric mean	Mean (above the MIC)[b]	SD
CT-WT1	0.25 to 0.5	0.25	0.31	23.0	8.1
CT-WT2	0.25 to 0.5	0.25	0.29	13.4	2.4
CT-TR1	0.25 to >16	0.5	0.45[c]	34.6	11.9
CT-TR2	1 to >16	>16	ND[d]	50.1[b]	10.4
CT-TR-R	>16	>16	ND	71.8[b]	5.4
CT-R	8 to 16	16	ND	ND	ND

[a]Values are ranges, modes, and geometric means from seven repetitions.
[b]As isolates CT-TR-R and oftentimes CT-TR2 had MICs >16 mg/liter despite a flat growth curve, the percentage was calculated for all wells above the FLC susceptibility breakpoint of 2 mg/liter.
[c]Excluding one of seven isolates with a growth curve crossing the 50% inhibition line twice.
[d]ND, not determined.

was observed compared to that in both CT-WT1 and CT-WT2 (Fig. 2). All isolates were *ERG11* wild types.

***In vivo* studies using the *Galleria mellonella* model.** Mortality in the larva model was >90% by day 2 in all placebo groups and with a maximum of one larva still alive on day 5. A clear dose-dependent survival was observed, especially for isolates CT-WT1 and CT-TR1 (Fig. 3). The survival rates for larvae challenged with CT-WT1 were >50% and >70% at day 5 in treatment groups receiving 5 and 20 mg/kg, respectively. In contrast, the survival was 40% for CT-TR1 and 10% to 20% for the remaining isolates in the 20 mg/kg groups. Given the pH-dependent trailing demonstrated *in vitro*, the pH values in hemolymph of uninfected versus infected larvae were investigated but were found to be similar and only slightly acidic (pH 6.6 versus 6.5).

***In vivo* studies using the immunocompetent murine model over 96h.** The kidney burden was lower in mice treated with 35 mg/kg fluconazole than in untreated mice challenged with all isolates except CT-TR-R ($P = 0.56$) (Fig. 4A). A dose-dependent reduction was observed, and 15 mg/kg was significantly better than no treatment for

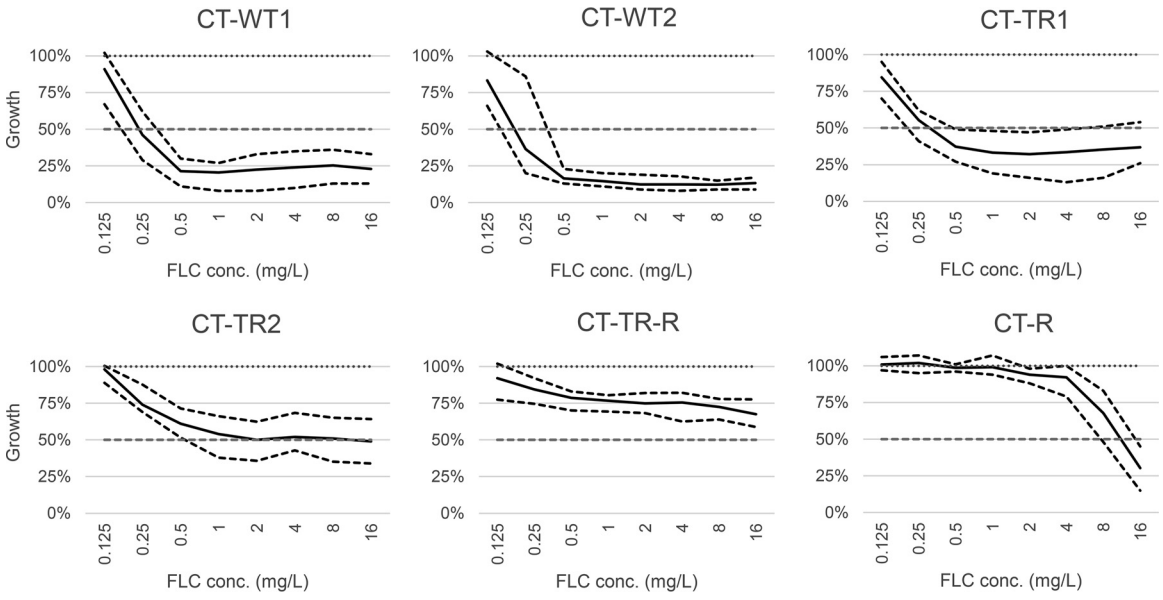

FIG 1 Overview of the EUCAST microdilution enzyme-linked immunosorbent assay (ELISA) readouts of the phenotypes of the six included isolates. Means (solid black lines) and ranges (dotted black lines) of seven repetitions as well as the 50% and 100% (antifungal-free control) growth lines (gray dotted lines) are shown. The background OD of the ELISA reader has been subtracted. On the *y* axes, growth in individual wells is presented as percentages of growth compared to growth in the antifungal-free growth control wells. For the ranges, the repetitions with the most and the least degrees of trailing were chosen.

TABLE 2 Effect of different pH values of the medium on EUCAST susceptibility results[a]

	MIC (mg/liter)			% residual growth[b]		
Phenotype	pH 7.5	pH 6	pH 4.5	pH 7.5	pH 6	pH 4.5
CT-WT1	0.125	0.25	0.5	4	3	10
CT-WT2	0.25	0.5	1	3	12	13
CT-TR1	0.125	0.25	0.5	3	1	11
CT-TR2	0.25	0.5	2	20	32	48
CT-TR-R	**>128**	**>128**	**>128**	**80**	**70**	**79**

[a]Bold numbers refer to residual growth of ≥50% or to MIC values classified as resistant.
[b]The proportion of trailing in the 8 mg/liter concentration well compared to the antifungal-free growth control.

all isolates except CT-TR1 ($P = 0.08$) (Fig. 4B). In isolate CT-WT2, the 5-mg/kg dose resulted in significant efficacy compared to untreated animals.

***In vivo* studies using neutropenic model over 24 h.** Fluconazole 15 and 35 mg/kg were significantly more efficacious than no treatment for mice challenged with wild-type (CT-WT1 and CT-WT2) and moderate (CT-TR1 and CT-TR2) trailing. Only a treatment with 35 mg/kg was better than no treatment for the CT-TR-R isolate ($P = 0.04$) (Fig. 5). As expected, no treatment dose was effective for infection caused by the CT-R isolate (Fig. 5).

DISCUSSION

A stepwise reduction in fluconazole efficacy was found for isolates with increasing degrees of trailing in the *G. mellonella* larva lethal candidiasis model, suggesting the observed differential susceptibility patterns were not solely an *in vitro* phenomenon. However, in immunocompetent mice, only the heavily trailing and fully resistant isolates failed to respond to fluconazole. We first speculated that the involvement of the murine immune system contributed to this discrepancy and repeated the experiments in the immunosuppressed mouse model. However, also in the case of the immune-suppressed model, only heavily trailing or the strictly resistant isolates were associated with a reduced response to fluconazole.

Murine models of invasive candidiasis are considered gold standards of *in vivo* animal candidiasis experimentation due to the similarity between humans and mice regarding internal anatomy, immune system, metabolism, and body temperature. However, several insect models have been of value in the testing of pathogenicity and treatment response to fungal infections. These models offer advantages in terms of low cost, ease of handling, an intact innate immune system, and a lack of ethical constraints (27–29). The *G. mellonella* model is attractive due to its sufficient size for enabling

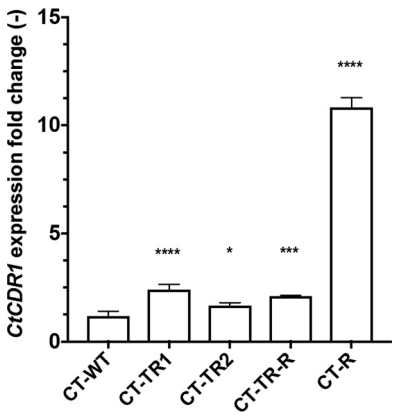

FIG 2 Expression levels of *CDR1* in *C. tropicalis*. Expression levels were calculated as described in Materials and Methods and were normalized to *ACT1* expression relative to merged data of CT-WT1 and CT-WT2 (CT-WT). ANOVA one-way statistics using merged data for *CDR1* of both CT-WT1 and CT-WT2 (CT-WT) were employed as a reference. NS, not significant; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

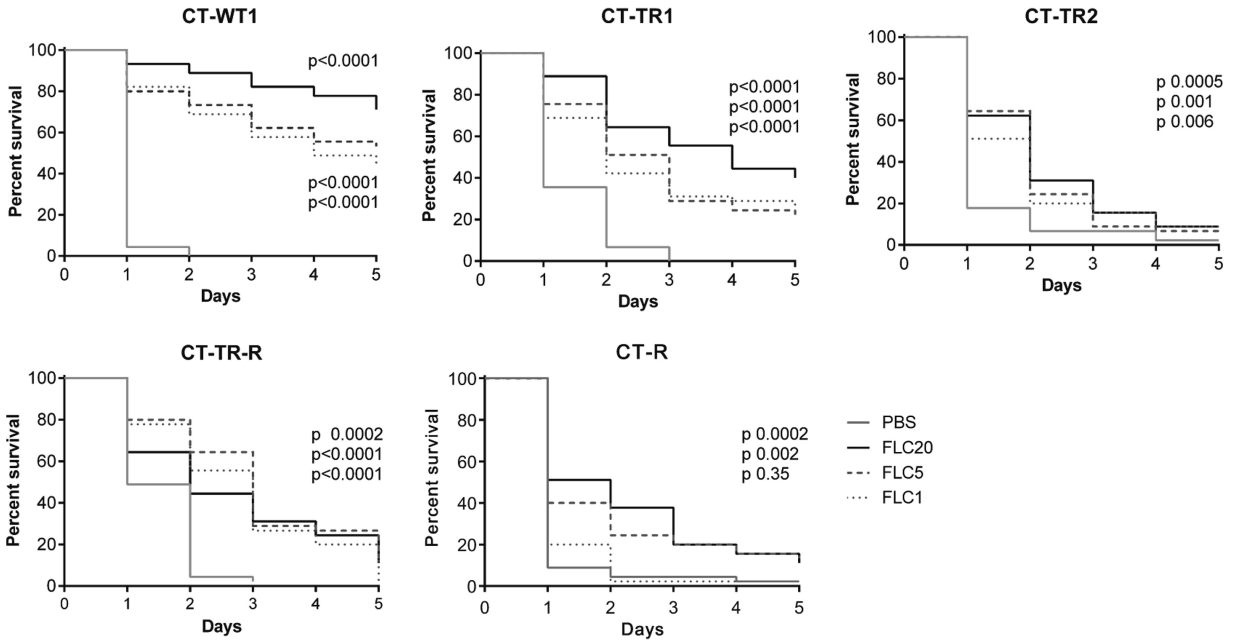

FIG 3 Kaplan-Meier curves of larval survival over 5 days depending on treatment for the five included isolates. PBS, solid gray line; FLC 1 mg/kg, dotted line; FLC 5 mg/kg, dashed line; FLC 20 mg/kg, solid black line. The *P* values are results from the log rank test of PBS versus treatment groups (the *P* values on top are for PBS versus FLC 20 mg/kg and values on the bottom are for PBS versus FLC 1 mg/kg).

precise challenge and treatment injections to be administered. Furthermore, the larval temperature tolerance enables experiments to be conducted at body temperature, which is important for the expression of some virulence traits (30). Notably, previous *Galleria* experiments demonstrated that *C. tropicalis* infection causes tissue invasion and mortality with a fluconazole dose- and MIC-dependent response (24, 26). We chose an upper dose of 20 mg/kg, which is well tolerated and gives a 24-h area under the curve (AUC) exposure of 400 mg·h/liter. This dosage corresponds to the human exposure during standard licensed dosing (31, 32).

The discrepant findings between the two *in vivo* models are intriguing given the

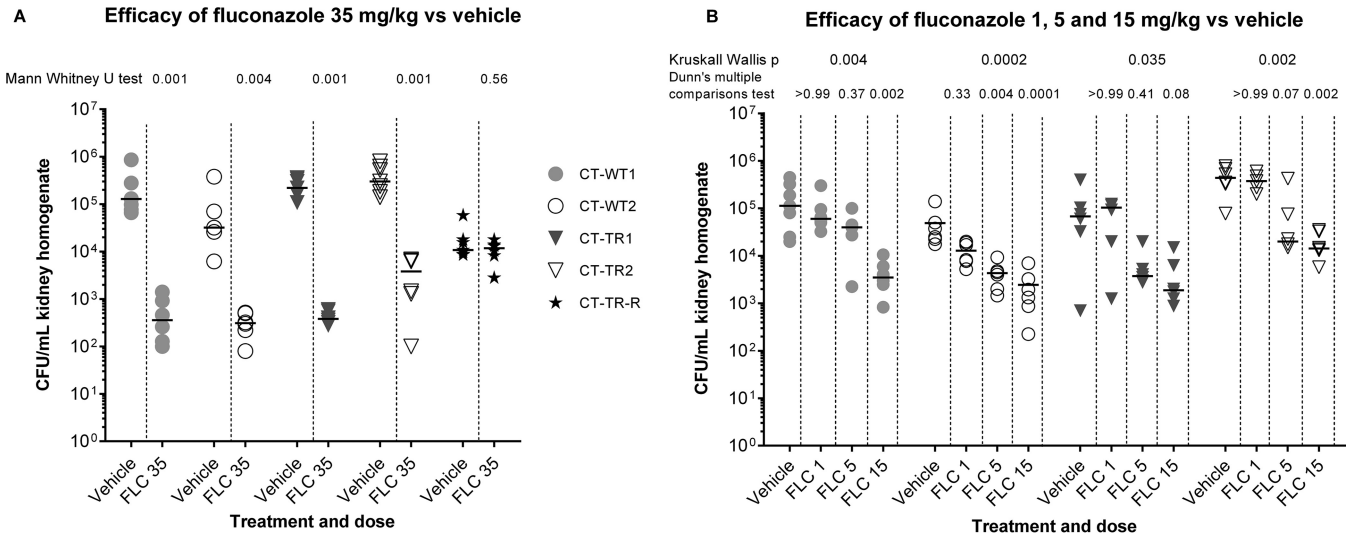

FIG 4 Treatment efficacy over 96 h of various doses of fluconazole on *C. tropicalis* infection with trailing and nontrailing isolates in an immunocompetent mouse model. Horizontal lines indicate the median values. (A) CFU counts on day 4 after inoculation and daily treatment (starting at $t = 2$ h) with one dose of either vehicle control or fluconazole 35 mg/kg. (B) For isolate CT-TR-R, no reduction in CFU counts was found. For the remaining four isolates, lower dosages of fluconazole 1, 5, and 15 mg/kg were also subsequently tested.

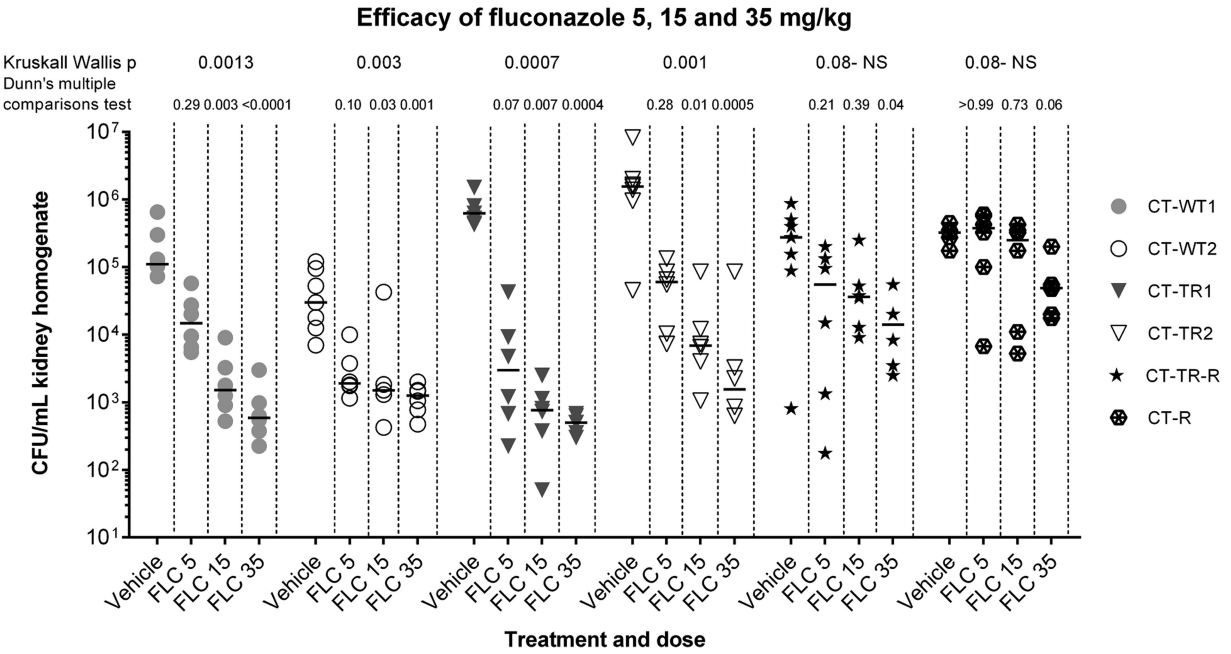

FIG 5 Treatment efficacy over 24 h of various doses of fluconazole on *C. tropicalis* infection with trailing and nontrailing isolates in a neutropenic mouse model. Horizontal lines indicate the median values. One pair of kidneys was lost during transport between facilities prior to CFU determination (CT-WT2, FLC 15 mg/kg) and thus was not available for analysis.

substantial *in vitro* growth at higher fluconazole concentrations for trailing isolates. The *in vitro* trailing was potentiated with a decreasing pH. This is in contrast with results from a previous report (15) but raise questions as to whether fluconazole exhibited a reduced efficacy against trailing isolates in the larvae due to the slightly lower pH and whether drug efficacy may be affected when low pH foci are present.

Due to ethical constraints, we were not able to apply a mortality endpoint to the murine experiments. It is possible that the lethal infection in the *G. mellonella* larvae exacerbated subtle differences in susceptibility, especially given that fluconazole was only administered once. In contrast, mice were administered daily doses initiated as early as 2 h postchallenge for a less severe infection followed by fungal load determinations. Infections caused by isolates with variable susceptibilities may be resolved due the intervention of immune responses. This type of response is important for efficient clearance of fungal pathogens, especially when exposed to fungistatic drugs (33, 34). In immunocompetent mice, some individuals may partially clear the infection, hypothetically compensating a decrease in drug activity. However, the 24-h experiments using immunosuppressed mice did not consistently support this hypothesis. Nevertheless, our data suggest that trailing growth, at least when it is prominent (growth $>$50% above the endpoint reading), impacts drug treatment efficacy in all models.

This study has limitations. Even though the comprehensive molecular trailing phenotype remains uncharacterized, our results indicated that in the absence of involvement of target gene *ERG11* mutations, fluconazole resistance in the CT-R isolate may be explained by the strong upregulation of *CDR1*. This also was the only isolate that was strictly azole resistant when adopting the 50% susceptibility testing endpoint. Of note, *CDR1* expression distinguished low and high trailing isolates from the wild-type (WT) isolates. However, *CDR1* expression was not related to the degree of trailing among the isolates (Fig. 2); therefore, we speculate that other mechanisms may play in concert (e.g., *ERG11* and/or *MDR1* upregulation) (20–22). If it is mediated by efflux pump upregulation or another inducible mechanism, a 24-h murine model experiment may not be appropriate to detect resistance dependent on induction. Another potential caveat was that concomitant administration of ibuprofen was mandatory during murine experimentation. Ibuprofen has been shown to potentiate the *in vivo* activity of

fluconazole against resistant *C. albicans* in a murine infection (35). When retesting the trailing isolates in the neutropenic 24-h model without ibuprofen, fluconazole efficacy remained unchanged (data not shown).

In conclusion, the undertaken *in vivo* experiments yielded differential results dependent on the type of animal model. In the lethal *Galleria model*, fluconazole efficacy was inversely correlated with the degree of early 24-h trailing, whereas no lack of fluconazole efficacy was observed in the nonlethal murine models except for isolates with heavy trailing (>50% growth compared to that of the control). The data presented here indicate that both the degree of trailing and type of *in vivo* model are critical for predicting fluconazole treatment failure. Until further data are available, heavily trailing isolates should be regarded as clinically resistant to fluconazole, whereas lower degrees of trailing, depending on the model used, appear to have little or no impact on fluconazole efficacy. Thus, our findings *per se* support the use of a 50% cutoff as the endpoint for susceptibility categorization, at least in the nonseverely ill population, in agreement with previously published reports (5, 13). However, the findings that *CDR1* expression is upregulated in trailing isolates and that fluconazole efficacy is lower in the lethal *Galleria* model suggest that caution may be warranted when contemplating fluconazole step-down treatment for severe infections, especially in fragile or immunocompromised patients. This concern is supported by a recent study documenting fluconazole failure in the *Galleria* model of fluconazole trailing/tolerant *C. albicans* isolates from patients with persistent but not resolving candidemia (36). Further characterizations of the mechanisms behind trailing in *C. tropicalis* and the potential implication in severe infections are warranted.

MATERIALS AND METHODS

Ethics. Murine experiments were conducted in accordance with the recommendations of the European community (directive 2010/63/EU, 22 September 2010) and were approved by the Animal Experiments Inspectorate under the Ministry of Environment and Food of Denmark (number 2014–15-0201-00374). There are no legal requirements regarding *Galleria mellonella* experimentation.

Isolates. Six clinical isolates of *C. tropicalis* were selected among stored isolates to cover a range of azole EUCAST susceptibility phenotypes. The isolates included were CT-WT1 and CT-WT2 with weak trailing (<25% mean trailing growth), CT-TR1 and CT-TR2 with moderate trailing (26% to 50% mean trailing), and CT-TR-R with heavy trailing (>70% of mean trailing) resistance, and finally one resistant (nontrailing) isolate, CT-R, displaying uninhibited (100%) growth at concentrations of ≥4 mg/liter. Isolates were grown on CHROMagar (CHROMagar Co., Paris, France) for purity control and subcultured on Sabouraud dextrose agar (pH 4) (SSI Diagnostika, Hillerød, Denmark) at 35°C for 24 to 48 h prior to inoculation.

***In vitro* susceptibility testing.** EUCAST MICs for fluconazole (Sigma-Aldrich, final drug concentration range of 0.125 to 16 mg/liter) were determined according to the E.DEF 7.3 (10). The degree of trailing was determined as the percentage growth in wells with concentrations above the MIC compared to the growth control (background optical density [OD] subtracted). For isolates with an MIC of >16 mg/liter due to a trailing phenotype (CT-TR-R and occasionally CT-TR2), the percentage of trailing was determined for wells with concentrations at or above the susceptibility breakpoint of 2 mg/liter. The presented EUCAST MICs and trailing are mean results from seven tests on different days and batches of susceptibility plates. Finally, the effect of medium pH was examined using RPMI 1640 medium (Sigma-Aldrich) buffered by 0.1 M MOPS (morpholinepropanesulfonic acid) at pH 4.5, 6, and 7.0 using either HCl or NaOH.

Reverse transcription-quantitative PCR. Total RNA was extracted with a phenol-chloroform-lithium chloride procedure as previously described (37). Biological triplicates from strains were prepared by growing the strains in 10 ml of yeast extract-peptone-dextrose (YEPD) medium until mid-exponential-growth phase (1.5×10^7 cells/ml). Contaminating genomic DNA was removed by DNase treatment (DNA free; Ambion). The expression of *CDR1* was quantitatively determined by quantitative real-time reverse transcription-PCR in a StepOne real-time PCR system (Applied Biosystems). The expression levels were normalized to *ACT1* expression. Changes (*n*-fold) in gene expression relative to that of both strains CT-WT1 and CT-WT2 were determined from *ACT1*-normalized expression levels. The primers and probes used for this assay for *CDR1* were CDR1F, GAGGTGTTTCTGGTGGTGAA; CtCDR1R, GCAGCATCCAAACCTC TAGT; and CDR1P, TGCTGAAGCATCCTTAAGTGGTGCT; and for *ACT1* were ACT1F, CTCCTTGCCACACGGT ATTT; ACT1R, GGTGGTGGAGAAAGTGTAACC; and ACT1P, TGGCTGGTAGAGACTTGACCGACT. The primers used as TaqMan probes were labeled with 6-carboxyfluorescein (6-FAM) at the 5′ end and with tetramethylrhodamine (TAMRA) at the 3′ end.

***ERG11* sequencing.** DNA was extracted from pure cultures using the NucliSENS easyMag (bio Mérieux) according to the manufacturer's instructions. The *ERG11* gene was amplified in duplicates by conventional PCR with 40 cycles at an annealing temperature of 58°C, including an initial denaturation at 94°C and subsequent 72°C extension for 60 s. The primers used are listed in Table S1 in the

supplemental material. All PCR products were sequenced by Macrogen, Holland, and compared to the *ERG11* wild-type sequence from *C. tropicalis* reference strain ATCC 750 (GenBank accession M23673).

***Galleria mellonella* model.** Sixth-instar *G. mellonella* larvae (HPreptiles, Copenhagen, Denmark) were used, stored, weighted, and washed as previously described (32). The mean weight of treatment groups was used for antifungal drug dosing. An individual experiment consisted of 15 larvae/treatment group (phosphate-buffered saline [PBS] and fluconazole 1, 5, and 20 mg/kg larvae) for all isolates (except CT-WT2, which was acquired later and only included in the murine experiments). Inocula previously determined to achieve an infection causing 100% mortality in PBS-treated larvae within 3 days were used. Yeast suspensions were prepared from Sabouraud agar by adding 24-h colonies to PBS (SSI Diagnostika, Hillerød, Denmark) with 20 mg/liter ampicillin (Pentrexyl; Bristol-Meyers Squibb, Virum, Denmark) for a final concentration between 0.5×10^8 and 1×10^8 CFU/ml. The concentrations were controlled by the spot technique (38). Larvae were inoculated in the last left proleg with 10 μl of yeast suspension (500-μl Hamilton syringe with a PB600-1 Repeating Dispenser). After 28 ± 3 min, the larvae received 10 μl of control (PBS) or fluconazole treatment (1, 5, or 20 mg/kg larvae) prepared by diluting fluconazole (2 mg/ml for intravenous use; Fresenius Kabi) with sterile isotonic NaCl (B. Braun Melsungen AG, Melsungen, Germany) in the last right proleg. Control groups received fluconazole 20 mg/kg, PBS, NaCl, or PBS and NaCl to check for excessive death by any one agent or dual injections. For the final analyses, the results from three individual experiments were pooled (45 larvae/group). Only experiments with similar degrees of mortality in the PBS group ($\geq$14 larvae on the third day) were included. Mortality in the unchallenged control larvae never exceeded the predetermined limit of 15%.

To assess if the pH of the infected and noninfected larvae were different and might influence the degree of trailing in infection, the pH values of hemolymph from larvae without infection (pooled hemolymph from 20 larvae [275 to 325 mg/larva]) and early and late infection were measured (PHM 92; Radiometer Copenhagen, Denmark). For the early and late infection, larvae were inoculated with 10 μl of a suspension of 6.5×10^6 CFU/ml of CT-TR2, followed 30 min later with 10 μl of fluconazole 20 mg/kg. The hemolymph was pooled from 20 larvae sacrificed 1 h posttreatment (for early infection) and from the 13 surviving of 30 infected larvae 22 h posttreatment (for late lethal infection).

Murine models. (i) Mice. NMRI mice (weight, 26 to 30 g; Taconic, Lille Skensved, Denmark) were used throughout the study. Groups of 3 to 4 mice were used for the virulence studies and of 6 to 7 mice for treatment groups. Ibuprofen 30 mg/kg bodyweight (Nurofen Junior 20 mg/ml; Reckitt Benckiser Deutschland GmbH, Germany) was administered once daily to minimize discomfort and pain (per requirement).

(ii) Immunocompetent model. On the basis of a prior virulence study (not shown), an inoculum ($\approx 1 \times 10^6$ to 5×10^6 CFU/mouse) resulting in a nonlethal sustained kidney infection was chosen for each of the five included *C. tropicalis* isolates (CT-WT1, CT-WT2, CT-TR1, CT-TR2, and CT-TR-R). On day 0, groups of mice were inoculated in the lateral tail veins with 200 μl of *Candida* suspension. Treatment was administered intraperitoneally once daily (starting 2 h postinoculation) for four doses of 0.5 ml vehicle (NaCl) or fluconazole 35 mg/kg (2 mg/liter diluted in sterile saline; Fresenius Kabi). Mice were humanly sacrificed on day 4 (after 96 h of treatment), and the kidneys were harvested. For isolates CT-WT1, CT-WT2, CT-TR1, and CT-TR2, a second similar experiment was conducted with vehicle and treatment groups of 1, 5, and 15 mg/kg fluconazole. Mice displaying discomfort or inanition during the study were sacrificed by cervical dislocation. The actual inoculum was slightly higher than expected for isolate CT-WT2 (7.0×10^6 CFU/ml). Two mice infected with this isolate were euthanized (one in the vehicle group on day 1 and one in the 1 mg/kg fluconazole [FLC1] group on day 2), and the resulting CFU counts were excluded from subsequent analyses. The remaining inocula were 2.2×10^6 to 5.5×10^6 CFU/ml.

(iii) Immunosuppression model. For this model, mice received two doses of cyclophosphamide (Sendoxan; Baxter) intravenously (200 mg/kg on day -4 and 100 mg/kg on day -1), yielding them neutropenic for the duration of the experiment (33, 39, 40). Virulence studies were conducted to determine an appropriate inoculum of each of the six included *C. tropicalis* isolates (CT-WT1, CT-WT2, CT-TR1, CT-TR2, CT- TR-R, and CT-R). No inoculum was determined to enable a sustained nonlethal infection over 48 h; however, approximately 0.5×10^5 to 1×10^5 CFU/ml could be used over 24 h. Mice were inoculated with 200 μl of yeast suspension intravenously and then 2 h later with 0.5 ml intraperitoneally of vehicle (NaCl) or fluconazole diluted to concentrations corresponding to 5, 15, or 35 mg/kg. Mice were sacrificed 24 h posttreatment.

For all experiments, the kidneys were aseptically harvested and frozen pairwise at -80°C. After thawing, they were homogenized by bead beating with two stainless steel beads in a Tissue Lyser II (Qiagen, Denmark) for 2 min at a frequency of 30/s after the addition of 1 ml of sterile water, and were serially diluted for CFU determination using the spot technique. One pair of kidneys was lost during transport between facilities prior to CFU determination (CT-WT2, fluconazole [FLC] 15 mg/kg, ibuprofen treated) and was not available for analysis.

Statistics. (i) *Galleria mellonella*. For individual isolates, the treatment effect was assessed with a log rank test.

(ii) Mouse models. CFU counts of individual isolates were compared between control and treatment groups by using the Mann-Whitney U test or Kruskal-Wallis (where appropriate) test with Dunn's multiple-comparison test.

(iii) *CDR1* expression. Merged CT-WT1 and CT-WT2 data were compared to other expression data using a one-way analysis of variance (ANOVA) with a Dunnett's multiple-comparison test. All statistical calculations were performed using GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA, USA). For all analyses, a P value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01624-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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